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Enzymology of Protein Methylation

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INTRODUCTION

A little more than a dozen protein posttranslational side-chain modification reactions have so far been identified, and they constitute one of the most rapidly developing and exciting frontiers in the modern biological sciences. One such side-chain modification reaction, *Protein Methylation,* occurs ubiquitously in nature, in organisms ranging from prokaryotic to eukaryotic, and involves N-methylation of arginine, lysine, histidine, alanine, proline, and glutamine, and O-methylation of glutamic and aspartic acid (Paik and Kim 1980, 1975). Among these amino acid residues, however, the enzymatic methylation of lysine, arginine, histidine, and dicarboxylic amino acid residues have been most actively investigated (Table 1).

In nature, methylated amino acids occur in highly specialized proteins such as histones, flagella protein, myosin, actin, ribosomal proteins, opsin, EF 1α, HnPNP protein, HMG-1 and HMG-2 protein, fungal and plant cytochrome c, myelin basic protein (Al basic protein), EF-Tu, porcine heart citrate synthase, heat-shock protein, nucleolar protein, wheat α-amylase and calmodulin (Table 2).

During the past 15 years, it has been revealed that these methylations are carried out by several classes of highly protein-specific methyltransferases (Table 1) (Paik and Kim 1968, 1970; Kim and Paik 1970). For example, protein methylase I [S-Adenosyl-L-methionine: protein-arginine N-methyltransferase; EC 2.1.1.23] representing one such class methylates the guanidino group of arginine residues, likewise protein methylase II [S-Adenosyl-L-methionine: protein-carboxyl O-methyltransferase; EC 2.1.1.24] methylates the carboxyl group of glutamyl or aspartyl residues, and protein methylase III [S-Adenosyl-L-methionine: protein-lysine N-methyltransferase; EC 2.1.1.43] methylates the ε-amino group of lysine residues (Table 1 and 3). At least several examples within each of these classes of methyltransferases have been well characterized. Furthermore protein methylase III has a counterpart enzyme ε-alkyllysinase [ε-Alkyl-L-lysine:oxido reductase; EC 1.5.3.4] that dealkylates methylated protein. In addition to these enzymes, evidence indicates the presence of an enzyme that methylates histidine residues of muscle protein. However, this enzyme has never been characterized nor solubilized (Table 1). In this chapter, some physicochemical properties and physiological significance of the aforementioned various enzymes are discussed.

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Enzymology of Protein-Arginine methylation [Protein Methylase I; EC 2.1.1.23]

A-Adenosyl-L-methionine:protein-arginine N-methyltransferase or protein methylase I [EC 2.1.1.23] was first isolated from calf thymus by Paik and Kim (1968). The enzyme is mainly found in the cytosol, however trace amounts have also been shown in the nucleus (Gallwitz 1971). Purified protein methylase I forms *in vitro* three products which have been identified as N^G-mono, N^G, N^G-di-(asymmetric), and N^G, N'^G-di-(symmetric) methylarginine (Lee *et al.* 1977). As shown in Table 2, many proteins are known to contain methylated arginines. The enzyme is found in many vertebrate tissues, however it is most abundant in brain, thymus, testis and spleen (Table 3). A form of protein methylase I has also just recently been isolated from wheat germ which is the first report of the enzyme being present in the plant kingdom.

Published reports have shown that protein methylase I has been partly purified from various sources; rat and calf brain, calf thymus, krebs ascites tumor cells and chicken embryo fibroblasts (Table 4). Wheat germ protein methylase I has several distinctive properties from animal protein methylase. These differences include optimum pH, relative ratio of *in vitro* reaction products, protein substrate specificity, inhibition by adenosine and the requirement of a peptide cofactor for full enzyme activity.

Table 1. Protein methyltransferases

	Trivial Name	Systematic nomenclature (E.C. number)	Methylation Product Amino acid and product	Stability
N-methylation	Protein methylase I	S-Adenosylmethionine: protein-arginine N-methyltransferase (EC 2.1.1.23)	Arginyl: N^G-monomethyl- N^G, N^G-Dimethyl- N^G, N'^G-Dimethyl-	Stable in 6 N HCl, but unstable in 0.2 N NaOH
	Protein methylase III	S-Adenosylmethionine: protein-lysine N-Methyltransferase (EC 2.1.1.43)	Lysyl: ε-Monomethyl- ε-N-Dimethyl- ε-N-Trimethyl-	Stable in both 6 N HCl and 0.2 N NaOH
		S-Adenosylmethionine: protein-histidine N-Methyltransferase	Histidyl: 3-N-methyl-	Stable in both 6 N HCl and 0.2 N NaOH
O-methylation	Protein methylase II	S-Adenosylmethionine: protein-carboxyl O-Methyltransferase (EC 2.1.1.24)	Glutamyl: Methyl ester Aspartyl: Methyl ester	t1/2=30 min at pH=7.1 at 37°C
S-methylation		S-Adenosylmethionine: protein-methionine S-methyltransferase	Methionyl: S-Methylmethionine	Stable in acid but unstable in alkaline

Table 2. Natural occurrence of various methylated amino acids

Methylated amino acid residues	Proteins
ε-N-Monomethyllysine	Flagella protein, histones, myosin, actin, ribosomal proteins, opsin, tooth matrix protein, EF 1α (fungus *Mucor*)
ε-N-Dimethyllysine	Flagella protein, histones, myosin, actin, opsin, ribosomal proteins, EF 1α (fungus *Mucor*)
ε-N-Trimethyllysine	Histones, cytochrome *c* (*Ascomycetes*), myosin, actin, ribosomal proteins, calmodlin, EF-Tu, citrate synthase (porcine heart), heart-shock proteins, EF 1α (fungus *Mucor*), α-amylase (wheat)
N^G-Monomethylarginine*	Histones, acidic nuclear protein, Al basic protein, heat-shock proteins
N^G, N^G-Dimethylarginine	Histones, Al basic protein, myosin, HnRNP protein, ribosomal proteins, tooth matrix protein, HMG-1 and HMG-2 protein, nucleolar protein C23
N^G, N'^G-Dimethylarginine	Histone, Al basic protein, tooth matrix protein, myosin, actin opsin
3-N-Methylhistidine	Myosin, actin, histone, opsin
δ-N-Methylglutamine	Ribosomal protein
Glutamyl or aspartyl methyl ester	Membrane proteins of *Escherichia coli, Salmonella Typhimurium* and erythrocytes

* N^G-refers to the nitrogen of the guanidino group of arginine, and N^GG, N^G-dimethyl-arginine is dimethyl substituted on the same nitrogen atom in the guanidino group. Thus, this compound is also called asymmetric dimethylarginine. In the same terminology, N^G, N'^G-dimethylarginine is dimethyl substituted at two different nitrogen atoms and, therefore, called symmetric dimethylarginine.

Table 3. Distribution of various protein methylases in rat organs

Organs	Protein methylase*			ε-Alkyllysinase**
	I	II	III	
Brain	1.04	1.92	0.69	0.01
Testis	1.54	4.15	1.14	0.27
Thymus	0.96	1.04	3.21	
Spleen	0.67	0.89	1.98	0.26
Kidney	0.53	0.40	0.71	1.98
Heart	0.51	1.17	0.42	0.26
Pancreas	0.48			0.01
Liver	0.46	0.29	1.07	0.36
Muscle		1.52	0.05	0.01
Lung		0.80	1.21	0.01

* The activities of protein methylase I, II and III were assayed with histone type II-A (Sigma Chemical Co.) as a substrate, and expressed as picomoles of S-adenosyl-L-[*methyl*-^{14}C] methionine used per minute per mg of enzyme protein.

** Expressed as μl 0_2 consumed per hour per mg of enzyme protein.

A. Characteristics

1) Substrate specificity: The wheat germ protein methylase I is highly specific towards substrate protein (Table 4) (Gupta *et al.* 1982). Histones appear to be the only substrate for the wheat germ enzyme *in vivo* as well in *in vitro*. On the other hand, both histones and myelin basic protein (Al basic protein or MBP) serve *in vitro* as substrates for the calf and rat brain protein methylase I. However, recent evidence indicates that there are two distinctly different enzymes for methylation of histone and MBP, and this will be discussed more in detail later.

2) *In vitro* products: The question whether one or more than one enzyme is involved in this synthesis of N^G-mono-, N^G, N^G-di-, and N^G, N'^G-dimethylarginine residues remain open at present. Uneven distribution of these arginine derivatives in nature suggests that more than a single enzyme is responsible (Table 2). On the other hand, the ratios of the various methylated arginines formed by protein methylase I preparations during the 120-fold enzyme purification were found to be quite constant (Gallwitz 1971; Lee *et al.* 1977), strongly suggesting that a single enzyme is responsible for the formation of all three methylated arginines. A similar conclusion for the synthesis of the three ε-N-methyllysines has also been derived (Durban *et al.* 1978).

3) Structural requirements: Structural requirements of protein methylase I towards substrate protein remains unsettled. Baldwin and Carnegie (1971) earlier observed that crude rabbit brain protein methylase I *in vitro* methylates only a single residue at position 107 among a total 19 arginine residues of human MBP. This position of Res-107 arginine is partly methylated *in vivo* (more detailed description on the structure of MBP is described later). When the synthetic pentapeptide Lys-Gly-Arg-Gly-Leu, which constitutes the amino acid sequence of residues 105 to 109 of human MBP, was examined (Schafer 1974), it was found that this peptide did not serve as a methyl-acceptor for the enzyme. These observations indicate that not only the amino acid sequence, but also the length of the peptide as well as the the tertiary structure of the protein molecule, may play important roles in the structural requirements of the substate protein.

4) Kinetic parameters: The pH optimum for the wheat germ enzyme is about 9.0, which is significantly different from those of the enzymes isolated from mammalian and avian tissues (Table 4). The K_m values of the wheat germ and calf brain enzymes for S-adenosyl-L-methionine are 5.7×10^{-6} M and 2.1×10^{-6} M, respectively. The K_m values for histone with the wheat germ and calf brain enzymes are 2.5×10^{-5} M and 5.5×10^{-4} M, respectively.

5) Cofactor: What germ protein methylase I requires a low molecular weight cofactor for its enzyme activity (Gupta *et al.* 1982). The cofactor is dialyzable, heat-labile, and the stimulatory activity is destroyed by trypsin treatment, indicating that the cofactor is peptide in nature. Although not well defined, a similar cofactor requirement was also observed for calf thymus protein methylase I.

6) Inhibitors: Another unusual property of wheat germ protein methylase I is that the enzyme has a natural inhibitor. This is observed during the purification of the enzyme when an unusually high yield of activity is obtained (90-fold enrichment with a yield of 160% (Gupta *et al.* 1982). The wheat germ enzyme appears to be much more sensitive to the inhibitor when compared with that of calif brain. The purified natural inhibitor appears to be identical to adenosine, since the two compounds have identical UV absorption spectra, retention time on high performance liquid chromatography and pI value (Gallwitz 1971; Edgar and Hope 1974).

Wheat germ as well as Krebs II ascites cell and chicken embryo fibroblast protein methylase I have been extensively studied in respect to various S-adenosyl-L-methionine and S-adenosyl-L-homocyteine analogues (Enouf *et al.* 1979; Caesllas and Jeanteur 1978). For example, S-adenosyl-L-homocystein

Table 4. Properties of various protein methylase I's

Properties	Source of protein methylase I					
	Calf brain (7)*	Calf thymus (3)	Rat brain (9)	Krebs ascites cell (10)	Chicken embryo fibroblast (11)	Wheat germ (8)
Subcellular location	cytosol	cytosol	cytosol	cell sap	cytosol	cell sap
in vitro protein substrate	histone: MBP at 10:3	endogenous protein	histone:MBP at equal rate	histone	histone	histone
Purification achieved (-fold)	120	34	8	150	21	90
Molecular weight	–**	–		50,000	–	–
pH optimum	7.2	7.4	7.2	8.5	7.2	9.0
K_m for S-adenosyl-L-methionine ($\times 10^{-6}$ M)	7.6	2.1	0.53	2.5	8	5.7
K_m for protein substrate ($\times 10^{-5}$M)	2.5 for histone 7.1 for MBP	–	–	–	–	55
K_i for S-adenosyl-L-homocysteine ($\times 10^{-6}$ M)	2.62	–	0.27	1.4	8	1.05
Specific activity of purified enzyme***	38.3	17	–	20	183	14
pI value	5.1	–	–	–	–	–
Cofactor requirement	–	peptide	–	–	–	peptide
Ratio of mono, asymmetric di & symmetric dimethylarginine produced *in vitro*	55:5:40	–	3:1:1	–	–	73:27:0

* Reference number.
** Not reported
*** Picomoles of S-adenosyl-L-methionine used per min. per mg of enzyme protein.

A9145C, sinefungin and S-inosyl-(2-hydroxy-4-methylthio) butyrate are the most powerful inhibitors of the wheat germ enzyme with K_i values of 10^{-6} M to 10^{-8} M (Gupta *et al.* 1982). ATP, ADP and AMP are inactive as inhibitors, as well as other nucleosides and bases.

B. Biological Significance

1) Protein-arginine methylation and cell growth: Levels of protein methylase I activity, assayed with histone as a substrate, closely parallels the degree of cell proliferation. Elevated enzyme activity has been observed in regenerating adult rat liver (Lee and Paik 1972), rapidly growing hepatomas (Paik *et al.* (1975), fetal brain (Paik *et al.* 1972), and continuously dividing HeLa S-3 cell culture (Borun *et al.* 1972). Particularly during the hepatic regeneration, the *in vivo* [*methyl*-^{14}C] incorporation into histone-arginine residues preceded that of incorporation into histone-lysine residues by 24 hours, and the protein methylase I activity increased more than three-fold after four days of regeneration. The level of protein methylase I activity was also found to increase two- to nine-fold in the embryonic rabbit liver and mouse hepatoma (Turner and Hancock 1970). This enzyme activity paralleled closely the growth rate of Morris and Novikoff hepatomas, particularly in that the level of the enzyme activity in the cytosol of the fastest growing hepatoma was elevated approximately seven-fold (Fig. 1).

In continuously dividing synchronized HeLa S-3 cell culture, an average of approximately 12% of the radioactivity that was derived from L-[*methyl*-^{14}C] methionine and incorporated into H3 histone fraction, occuring at N^G-dimethylarginines (Borun *et al.* 1972). Furthermore, the peak of methylarginine synthesis coincides with that of DNA synthesis during the cell cycle. This observation stands in contrast to that of histone-lysine methylation that follows the peak of DNA synthesis. Therefore, although the biological significance of the increased protein methylase I activity and of the temporal relationship to DNA syn-

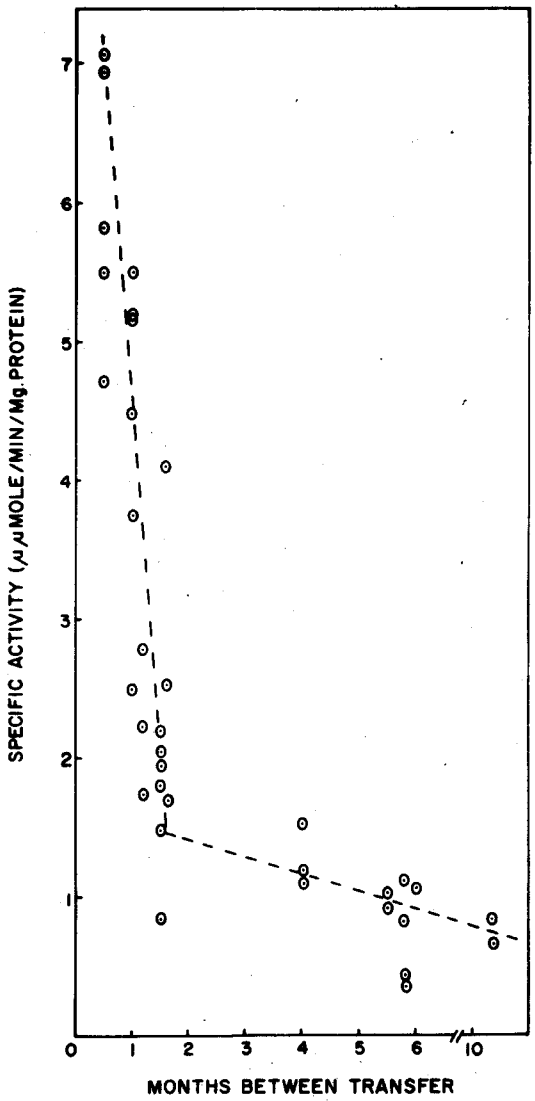


Fig. 1. Protein methylase I activity in hepatomas with varying growth rates.

thesis during the cell cycle is not obvious at present, the function of histone-arginine methylation is expected to be quite different from that of histonelysine methylation which has been postulated to play a role in the chromatin condensation prior to mitosis.

2) Methylation of MBP and myelin formation: The human MBP consists of 170 amino acid residues, and contains N^G-methylarginines at Res-107 (Baldwin and Carnegie 1971). Since the principal encephalitogenic determinant in the protein resides in residues 111 to 121, residue-107 methylarginine is quite close to the region of the determinant. The three proline residues located in the Res-99, -100 and -101 might bend the protein into a double chain. Therefore, Brostoff and Eylar (1971) suggested that the methylation of arginine at Res-107 could provide one of the sites of cross-chain stabilization of a double chain conformation, either by interaction with lipids or by conjugation with the adjacent phenylalanine side chain found in close proximity. A similar suggestion was also presented by Baldwin and Carnegie (1971) who stated that the arginine-methylation could be of aid in the conjugation of the myelin protein with the nonpolar lipid to form the myelin.

Thus, various investigators studied the protein methylase I activity during myelin formation in rats or mice, using histones as substrate, and came to the conclusion that there was no direct temporal correlationship between the protein methylase I activity and myelin formation in these animals (Jones and Carnegie 1974; Paik and Kim 1973). Recently, however, it has been realized that the assay conditions employed in the above investigations were not accurate in that a wrong substrate had been used. It is increasingly evident that the enzyme responsible for methylating histones is distinctly different from the MBP methylating enzyme (Lee *et al.* 1977; Gupta *et al.* 1982; Crang and Jacobson 1982): During 120-fold purification of protein methylase I from calf brain, the ratio of the enzyme activities determined with histones and MBP as substrates varied significantly (Lee *et al.* 1977), and the protein methylase I purified from wheat germ methylated only histones but not MBP (Gupta *et al.* 1982). Furthermore, Crang and Jacobson (1982) observed that, while the protein methylase I activity in the mice spinal cord with MBP as substrate increased between 8 and 45 days post-natal and correlated well with various parameters of myelination, the enzyme activity determined with histone as substrate rather decreased during this period of myelination.

However, the strongest indication of the critical role played by protein methylation in the integrity and maintenance of myelin comes from a series of recent observations on subacute combined degeneration (SCD). SCD is found in man with untreated vitamin B_{12} deficiency, and is characterized by degeneration of myelin sheaths of the spinal cord. Experimentally, SCD could be induced in mice (Dinn *et al.* 1980) or monkey (Scott *et al.* 1981) by exposing them to an atmospheric environment containing nitrous oxide (N_2O). When maintained in an atmosphere of 15% N_2O for about 10 weeks, monkeys become ataxic and the disorder progressed over a period of 2-3 weeks

until the animals were moribound (Scott *et al.* 1981). However, when their diet was supplemented with methionine, they were free of any detectable clinical changes. Microscopical examination of the spinal cord and peripheral nerves of the unsupplemented monkeys showed the classical changes of SCD and the histological changes correlated with the clinical observations. However, sections from the methionine-supplemented monkeys showed no change or only slight changes. Furthermore, Jacobson *et al.* (1973) showed that cycloleucine produced neurological changes in mice that were histologically indistinguishable from SCD. Cycloleucine is an analogue of methionine which inhibits its cellular transport and many methionine-dependent processes including the biosynthesis of S-adenosyl-L-methionine. S-Adenosyl-L-methionine is, of course, a biologically active methyl donor which methylates protein among other macromolecules.

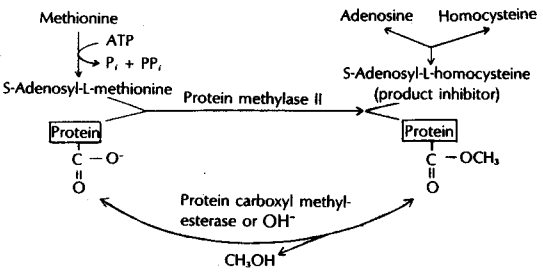
Inhibition of methylation of MBP might be responsible for the neurological damage caused by cycloleucine (Small *et al.* 1981; Ramsey and Fischer 1978). Cycloleucine strongly depressed methyl incorporation into N^G-methylarginines *in vivo*, experiments, Crang and Jacobson (1980) showed that cycloleucine caused a marked depression of S-adenosyl-L-methionine synthetic activity in brains of mice and that it also inhibited the methylation of MBP.

3) S-Adenosyl-L-homocystein analogues on protein methylase I and cell transformation: Recently, Casellas and Jeanteur (1978) investigated the effect of alteration of various regions of the S-adenosyl-L-homocysteine molecule, which is an extremely effective competitive inhibitor for S-adenosyl-L-methionine (Table 4). One interesting compound that emerged from this investigation was S-isobutyl adenosine (SIBA). The K_i value for this compound with partially purified Krebs II ascites cell protein methylase I was the lowest (0.15 mM) among the many transmethylation reactions tested *in vitro*. Enouf *et al.* (1979) further investigated the effect of these analogues on the transformation of chicken embryo fibroblasts by Rouse sarcoma virus (RSV), and found that there was a direct correlation between the inhibition of protein methylase I activity and the inhibition of RSV-induced cell transformation: All the good inhibitors of protein methylase I activity strongly prevented the virus-induced cell transformation. the inhibitory effect of these compounds is highly specific for protein methylase I. For example, although the K_i value of SIBA with protein methylase I was 0.6×10^{-3} M with competitive inhibition of the cell transformation, protein methylase III [S-adenosyl-L-methionine: protein-lysine N-methyltransferase; EC 2.1.1.43] was not inhibited at all at the concentration of 1.25 mM. The above results, therefore, might suggest a role of protein methylase I in cell transformation.

4) Elevated urinary excretion of N^G-methylarginines: The amounts of both N^G, N^G-di- and N^G, N^G-dimethylarginines were significantly elevated in the urine of children with muscular dystrophy (Diliberto and Axelrod 1974). The increase of N^G, N^G-dimethylarginine (asymmetric) is much greater than that of N^G, N'^G-dimethylarginine (symmetric). This significant increase in the urinary excretion of dimethylarginines in these patients was highly specific since the urinary amount of 3-N-methylhistidine did not change, and no increased amounts of these methylated amino acids were observed in the urine of children with other types of muscular diseases and also patients with disuse muscular atrophy.

Enzymology of Protein-Carboxyl Methylation [Protein Methylase II; EC 2.1.1.24]

Free carboxyl groups of aspartyl and glutamyl side chains in proteins are enzymatically methylesterified by S-adenosyl-L-methionine:protein-carboxyl O-methyltransferase [EC 2.1.1.24; protein methylase II; protein carboxymethyltransferase] utilizing S-adenosyl-L-methionine as the methyl donor according to the following scheme.



The enzymatic reaction product, protein-carboxyl methyl ester, is highly alkalisensitive compared to protein methyl esters chemically prepared, giving rise to methanol (Kim and Paik 1970, Diliberto and Axelrod 1974; Kim and Paik 1976). Thus, the enzyme had been earlier called the "methanol-forming enzyme" (Axelrod and Daly 1965). However, later studies revealed that methanol is not a primary enzymatic product but rather a by-product released subsequent to base-hydrolysis of the protein methyl ester (Kim and Paik 1976; Gagnon *et al.* 1978; Kleene *et al.* 1977). Recently, protein carboxymethylesterase, an enzyme which is specific for the enzymatically formed protein methyl ester, has also been reported (Stock and Koshland

1978; Gagnon 1979).

The most prominent feature of this reaction is to neutralize an anionic charge of the acceptor protein by insertion of the methyl group, and it has therefore been suggested that modification of proteins by the methylation-demethylation cycle could reversibly control the function of the proteins. Indeed, this suggestion has been borne out with bacterial sensory systems; methylesterification of membrane-bound protein (methyl-accepting chemotactic protein or MCP) was shown to be the biochemical mechanism by which bacteria can adapt toward temporal gradients of chemoattractants and repellants (Springer *et al.* 1979; Springer and Koshland 1977). Subsequently, similar studies were extended in attempts to correlate protein-carboxyl methylation and leukotaxis (O'Dea *et al.* 1978; Pike *et al.* 1979) or sperm motility (Bouchard *et al.* 1980; Gagnon *et al.* 1979). Unfortunately, however, definitive evidence of an interrelationship as was observed in bacterial systems is still lacking in studying mammalian systems.

Thus far, experimental evidence indicates that carboxyl-methylation of proteins in mammalian systems are clearly distinct from methylation found in bacteria in terms of the properties of the enzymes involved, stability of the enzymatic products as well as possible biological functions. Mammalian enzymes generally exhibit a broad protein substrate specificity and a lower extent of methylation as compared with prokaryotic enzymes. Furthermore, the mammalian protein-carboxyl methyl esters are much less stable than those formed in the bacterial systems. Presently, there is not yet a clear-cut biological role of proteincarboxyl methylation assigned to mammalian systems.

The first carboxyl-methylated amino acid found in protein was glutamyl γ-methyl ester and comes from the work with bacteria (Kleene *et al.* 1977; Van Derwerf and Koshland 1977). Even though the identification of the enzyme in mammalian sources preceded long before that of bacteria, the identification of aspartyl β-methyl ester in human erythrocytes has been reported only recently (Janson and Clarke 1980). Presently, there are numerous hypotheses (Paik and Kim 1980) concerning the role of the protein-carboxyl methylation in eukaryotes. However, in this article, no attempts will be made to cover all of the systems. Instead, a few representative examples will be discussed.

A. Mammalian System

1) S-Adenosyl-L-methionine:protein-carboxyl O-methyltransferase (Protein Methylase II; EC 2.1.1.24):

(a) Enzyme and substrate specificity; The presence of the enzyme was first reported in the pituitary gland (Axelrod and Daly 1965), followed by calf spleen (Liss *et al.* 1969) and calf thymus (Kim and Paik 1970). Protein methylase II is shown to be located in the cytosol in a variety of cells, except the brain where a membrane-bound form has also been observed (Iqbal and Steenson 1976). The enzyme is present in all organs of the rat examined (Table 3), with the highest concentration in testis followed by brain, pituitary gland, erythrocytes, heart and muscle. Liver and kidney contain very low levels of activity. The low level of enzyme activity in the liver is accentuated by the fact that this organ contains a large amount of a membrane-bound natural inhibitor for the enzyme (Kim and Paik 1971). Although the exact nature of the inhibitor has not yet been established, evidence indicates that it is a polypeptide with ubiquitous distribution in various rat organs.

(b) Proteins that undergo carboxyl-methylation; *Pituitary polypeptides.* Many pituitary polypeptides from the anterior and posterior lobes are excellent methyl-acceptors for the enzyme (Diliberto and Axelrod 1974; Kim and Li 1979). *In vitro* methylation studies indicate that ACTH is the most highly methylatable substrate. The methylated amino acid residue in this polypeptide has been shown to be the glutamic acid Res-28 among 5 potential sites (Kim and Li 1976b). Lutropin and β-lipotropin (Kim and Li 1979a) are also very active methyl-acceptors.

A major posterior pituitary polypeptide, neurophysin, is also an excellent methyl acceptor (Edgar and Hope 1976). Electrophoretic analysis of the acceptor after incubation of the granular fraction of the gland with S-adenosyl-L-[*methyl*-^{3}H] methionine and purified protein methylase II showed that over 80% of the total methylated protein was a single band with a molecular weight of 11,000, suggesting its identity as neurophysin. Since this polypeptide is known to serve as a carrier protein for vasopressin (Breslow 1974), Edgar and Hope (1974) compared the efficiency of methylated and unmethylated neurophysin to bind ligand by the use of agarose-coupled vasopressin. The results showed no difference in the binding of vasopressin between these two polypeptides, possibly suggesting that the methylation and binding sites may be different.

Erythrocyte membrane proteins. In vitro studies carried out by incubating isolated erythrocyte membranes and erythrocyte protein methylase II in the presence of S-adenosyl-L-[*methyl*-^{14}C] methionine has indicated that two major integral polypeptides are methylated; glycophorin A (comigrates with band 3

on SDS/PAGE) and band 4.5 (Galletti *et al.* 1979). On the other hand, *in vivo* studies where intact unlysed erythrocytes were used, two different methylation patterns emerged depending on the methyl donor used (Ro *et al.* 1984): With S-adenosyl-L-[*methyl*-^{3}H] methionine, a pattern quite similar to that seen in the *in vitro* experiments was observed, whereas with L-[*methyl*-^{3}H] methionine as the methyl donor primarily cytoskeletal proteins rather than integral proteins were methylated (Freitag and Clarks 1981), namely bands 2.1, 4.1 and 3.

It has also recently been shown that methylation of membrane proteins increases with the age of the erythrocyte (Barber and Clarke 1983). This increase is thought to occur by an increase in the number of methylatable sites in the protein. Since racemization of certain amino acids occur during the ageing process (Master *et al.* 1978), it has been suggested that D-aspartyl residues may be active methylation sites for protein methylase II. Therefore, an increase in methylation of older cells may result from an increase in D-aspartyl residues. The subsequent hydrolysis of the D-aspartyl β-methyl ester generates the natural L-aspartyl isomer, providing a repair mechanism. This provocative hypotheses certainly merits further investigation.

Calmodulin. A ubiquitous calcium-binding protein, calmodulin, was also shown to be an exceptionally good methyl-acceptor for purified protein methylase II. Gagnon *et al.* (1981) showed that carboxyl-methylation of calmodulin reduced its capacity to activate CAMP phosphodiesterase and that the decrease in activity paralleled the extent of methylation. The K_m value for calmodulin is reported to be 6.5×10^{-5} M (Cox *et al.* 1979).

Vesicular proteins that are related to secretary processes. The first exocytotic organ that has been studied in the search for a possible role of protein-carboxyl methylation in the secretory process is bovine adrenal gland. Diliberto *et al.* (1976) observed that the adrenal medulla contained protein methylase II several times higher than in the adrenal cortex and that the medullary catecholamine-containing chromaffin vesicles had the highest concentration of methyl-acceptor proteins for the enzyme. When the separated particles (chromaffin granules) were purified on sucrose density gradients and methylated *in vitro* with prufied enzyme and S-adenosyl-L-[*methyl*-^{3}H] methionine, methylation occurred preferentially on the cytoplasmic surface proteins. Further studies using intract particles and isolated chromaffin membrane showed that carboxyl methylation of proteins on these membranes caused a release of about 67% of the methylated proteins. These results indeed suggest the involvement of protein-carboxyl methylation in the neutralization of charges on the surface of chromaffin vesicles and consequently in the release of surface proteins. Both phenomena may possibly be required for exocytosis.

To investigate the proteins being methylated, Gagnon *et al.* (1978) analyzed the membrane proteins by SDS/PAGE and showed two major methylated protein bands of M, 55,000 and 32,000. Brochardt *et al.* (1978) also isolated and characterized the methyl-acceptor protein from the adrenal chromaffin granules and its lysates, which was shown to be the highly acidic protein, chromagraffin A.

Other excretory organs that have been studied in search for a possible relationship between protein-carboxyl methylation and exocytosis are the parotid gland, pancreas, synaptosomes as well as the posterior and anterior pituitary glands (Stritt matter *et al.* 1978; Compillo and Ashcroft 1982; Eiden *et al.* 1982; Gagnon *et al.* 1978; Gagnon and Axelrod 1979).

(c) Assay and purification; Protein methylase II activity can be determined by two different approaches utilizing radiomethyl-labeled S-adenosyl-L-methionine. The first approach is to directly determine the amount of radioactivity transferred from S-adenosyl-L-[*methyl*-^{14}C] methionine into substrate protein by precipitation with trichloroacetic acid (TCA) (Kim *et al.* 1975). The methylated protein is repeatedly washed with TCA to remove unreacted S-adenosyl-L-[*methyl*-^{14}C] methionine, followed by treatment with chloroform: ether: ethanol mixture to remove methylated phospholipid. The second approach takes advantage of the alkali-liability of the enzymatically formed ester bond. The reaction product is hydrolyzed in an alkaline solution and the liberated radioactive methanol is extracted using isoamyl alcohol (Diliberto and Axelrod 1974; Jamaluddin *et al.* 1976) collected by distillation under reduced pressure and moderate temperatures (Kim and Paik 1971). The second method is only suited for assaying the enzyme activity when using small peptides or protein that are not precipitated by TCA. A combination of the direct and indirect methods has also been developed (Diliberto 1976). With this method, protein is first precipitated by TCA and the precipitates are subjected to alkaline hydrolysis to measure the released methanol from the protein-carboxyl methyl esters by extracting with isoamyl alcohol.

Since the optimum pH of the reaction varies depending on the methyl-acceptor proteins used, it is advised to study first the effect of pH when an untested protein is first used. The enzyme exhibits the

maximum activity at around pH 6 with γ-globulin, histone, ovalbumin and ribonuclease as substrates. The optimum pH with gelatin is at 7.4 (Kim and Paik 1971); with clathrin at 7.6; and with serum at around 7 (Kim 1974).

Earlier, several laboratories have purified protein methylase II utilizing conventional ion-exchange as well as molecular sieve chromatographic methods (Diliberto and Axelrod 1974; Iqbal and Steenson 1976; Bouchard *et al.* 1980; Kim 1974, 1973; Polastro *et al.* 1978). Since the enzyme is an acidic protein (Kim 1973), an anion-exchanger can be successfully utilized to purify the enzyme. However, a recently developed affinity-chromatographic method is simple and effective to obtain highly pure protein methylase II from several mammalian organs (Kim *et al.* 1978; Kim *et al.* 1983): The potent methyltransferase inhibitor, S-adenosyl-L-homocysteine which was covalently linked to Sepharose beads has proved to be a very effective gel to bind protein methylase II. Subsequent washing of the gel with a buffer containing the methyl donor, S-adenosyl-L-methionine, specifically elutes the enzyme. Further purification of the enzyme by gel-filtration yields over 95% pure enzyme as determined by SDS/PAGE (Kim *et al.* 1983).

(d) Properties of the enzyme; *Catalytic properties* The specificity for protein substrates (methyl-acceptor) is rather broad. Many naturally occurring proteins serve as methyl-acceptors, although there is no correlation between methyl-acceptibility and pI values of the acceptors (Kim and Paik 1970). The best substrates so far reported in the literature are ACTH (Kim and Li 1976b) followed by calmodulin (Gagnon *et al.* 1981), acetylcholine receptor (Kloog *et al.* 1980), β-subunit of lutropin (or follicle-stimulating hormone) and β-lipotropin (Kim and Li 1979a). γ-Globulin, ovalbumin, histones and native bovine pancreatic ribonuclease are moderately active as substrates and are often used as the model methyl-acceptors in the assay. As a rule, denatured proteins are far better methyl-acceptors; for example, gelatin (Kim and Paik 1971), F-P-100 (Kim and Paik 1970) and oxidized ribonuclease (Kim and Paik 1971).

Since methylation constitutes a macromolecular modification, the amino acid sequence around the methylatable site as well as the chain length of the polypeptide should be an important determinant for specificity. Employing ribonuclease (pancreatic) as a model substrate (Kim and Paik 1971) and generating seven peptide fragments with different chain length (Jamaluddin *et al.* 1976; Kim and Paik 1971), it was found that the K_m values for the peptides were inversely proportional to the chain length; the shorter the peptide length, the larger the K_m value, while the V_{max} values were related to the number of potential methyl-acceptor sites in the molecule. With proteins other than ribonuclease, wide ranges of K_m values were observed; 0.1 mM for histone, 0.34 mM for ribonuclease, 4.6×10^{-5} M for γ-globulin and 7.7×10^{-6} M for lutropin. The copolymer of L-gultamic acid and L-tyrosine, although not a methyl-acceptor, exhibited a non-competitive inhibitory activity (with corticotropin as a substrate) with a K_i value of 0.11×10^{-6} M (Oliva *et al.* 1979). Polyglutamic and polyaspartic acids are weak inhibitors; 50% inhibition at a concentration of 1.66×10^{-4} M and 2.2×10^{-4} M, respectively.

The enzyme is highly specific for S-adenosyl-L-methionine as the methyl donor. The K_m value for S-adenosyl-L-methionine is 8.7×10^{-6} M. S-Adenosyl-L-ethionine shows only a 2.4% donor activity when compared to the methionine derivative. Deaminated S-adenosyl-L-methionine analogue [S-inosyl-L-(2-hydroxy-4-methylthio) butyrate and S-inosyl-L-methionine] and the decarboxylated derivative [S-adenosyl-(5') 3-methylthiopropylamine] all do not act as a methyl donor. S-Adenosyl-L-homocysteine, the demethylated S-adenosyl-L-methionine, is a competitive inhibitor with a K_i value of $0.9\times10_{-6}$ M. The D-isomer of S-adenosyl-L-homocysteine, however, is inactive as an inhibitor (Oliva *et al.* 1979), Antifungal antibiotics, sinefungin and A9145C which are analogues of S-adenosyl-L-homocysteine, are potent competitive inhibitors with K_i values of 2.6×10^{-6} M and 0.4×10^{-6} M, respectively (Borchardt *et al.* 1979; Trivedi *et al.* 1982).

The reaction mechanism has been studied with highly purified calf thymus protein methylase II and bovine ribonuclease as the methyl-acceptor (Jamaluddin *et al.* 1975) Initial velocity patterns converging at a point on or near the extended abscissa were obtained with either ribonuclease or S-adenosyl-L-methionine as the variable substrate. Inhibition by the reaction product, S-adenosyl-L-homocystein, was linear competitive against both S-adenosyl-L-methionine and ribonuclease, the apparent inhibition constants being dependent on the concentration of the non-varied substrate. Although the reversal reaction was not studied because of experimental difficulties in isolating the unstable product, protein-carboxyl methyl ester, these results are consistent with a random mechanism for the enzyme in which in rate-limiting step may be the interconversion of the termany complexes and all other steps may be in equilibrium.

Molecular properties. The M, of the enzyme purified from various mammalian tissues is estimated

to be 25,000 by gel-filtration method (Kim 1974). The highly purified enzyme preparation exhibits several peaks on electrofocusing columns; observed pI values are 4.9, 5.5 and 6.0 from rat erythrocytes, 5.5, 6.0 and 6.2 from calf brain and 4.85 from calf thymus (Kim 1973). The enzyme purified from equine erythrocytes was found to be a glycoprotein containing 2% neutral hexose (Polastro *et al.* 1978). The amino acid composition of the equine erythrocyte anzyme indicates one cysteine residue per molecule, and this may be one of the catalytic sites, since the enzyme is extremely sensitive to oxidation and is inactivated by p-chloromercuribenzoate (Kim and Paik 1970). The enzyme activity, however, can be restored by treatment with 12 mM 2-mercaptoethanol.

2) Protein-carboxyl methylesterase: Whereas protein methylase II has long been actively studied in several laboratories, a demethylating enzyme for the protein-carboxyl methyl ester has only recently been observed. Protein-carboxyl methylesterase in mammalian tissues is specific for enzymatically formed protein-carboxy methyl esters, giving rise to the formation of methanol and the unmethylated protein (Gagnon 1979). The enzyme appears to be present in all tissues examined, with the highest concentration in the kidney. The esterase activity is particularly rich in the kidney cortex as compared to the papillary cells (Chene *et al.* 1982). The enzyme is active over a broad pH range. However, the assay was carried out at pH 5.2 in order to prevent spontaneous hydrolysis of the methyl ester. Enzyme activity is enhanced in the presence of detergent (0.1% Nonidet p-40), indicating the presence of membrane-bound enzyme in addition to the soluble enzyme.

Although the above report by Gagnon and his coworkers is convincing enough to suggest the presence of an esterase particularly specific toward enzymatically formed proteincarboxyl methyl ester, the enzyme has not yet been purified. Purification of the enzyme should be the next step in order to fully understand the significance of this enzyme.

B. Bacterial System

1) Protein-carboxyl methylation and bacterial chemotaxis: Bacterial chemotaxis is a behavioral response to a gradient change of chemoattractants or repellants. The response is a transduction phenomenon, whereby information is transferred from the bacterial sensory system to the motor system. In 1967, Adler and Dahl (1967) discovered that a methionine auxotroph of *Escherichia coli* was motile, but not chemotactic unless methionine was supplied exogenously. It was then found that methionine via S-adenosyl-L-methionine (Armstrong 1972; Larsen *et al.* 1974; Aswad and Koshland 1975) was needed for the methylation of a cytoplasmic protein, a necessary event for chemotaxis to occur (Kort *et al.* 1975). The methylated amino acid residue on the methyl-accepting chemotactic protein (MCP) has been identified as γ-glutamyl methyl ester (Kleene 1977; Van Derwerf and Koshland 1977). Since then, many laboratories have been studying this simple and interesting system. A narrow substrate specificity of the enzyme for MCP, a high degree of methyl incorporation into MCP, the relatively stable chemical nature of protein-carboxyl methyl ester and most notably several genetic evidences support the involvement of membrane protein-carboxyl methylation in bacterial chemotaxis.

The original studies carried out with enteric bacteria (*E. coli* and *Salmonella typhimurium*) indicate that the addition of an attractant to bacteria causes an increase in the methylation of MCP, while dilution of the attractant causes a decrease in the methylation of MCP or demethylation. Repellants have the opposite effect. However, a recent study with *Bacillus subtilis* has shown that in this gram-positive bacteria the addition of an attractant increases the demethylation of MCP (Goldman *et al.* 1982), quite contrary to the response for enteric gram-negative bacteria. In any case, the level of MCP methylation is correlated with chemotaxis of bacteria, or more precisely the adaptation process.

The MCP of *E. coli* have been resolved into three classes, MCP I, II and III (Silverman and Simon 1977; Springer *et al.* 1977 Hazelbauer 1981). These are the products of genes *tsr, tar* and *trg,* respectively. The 60,000 dalton proteins encoded by the *tar* and *tsr* genes were shown to be the primary receptors for aspartate and serine (Wang and Koshland 1980). Mutants in *tsr* do not respond to gradients of one group of compounds (serine, acetate, indole and leucine), mutants in *tar* are insensitive to a different group (aspartate, maltose, Ni^{2+}, Co^{2+}) and those in *trg* to yet a third group (galactose and ribose). These are independent complimentary pathways for the transmission of excitatory signals from their respective receptor groupings to the flagella motor. Adaptation to a stimulus transmitted through a given pathway results primarily in an increase or decrease in the methylation of that particular MCP.

In the absence of a chemotactic stimulus the MCP are methylated to a basal level. This level rises to a new plateau when an attractant is added and then falls to its original basal value when attractant is removed. Recently, each MCP was found to be

multimethylated (DeFranco and Koshland 1980; Chelsky and Dahlquist 1980; Engstrom and Hazelbaur 1980), thus a complexed banding pattern was observed on SDS/PAGE. Furthermore, the properties of the glutamic acid residues which become methylated after stimulation with an attractant differ from the properties of the residues methylated as a basal level. There seems to be a preferred order in which those residues are methylated and demethylated (Springer *et al.* 1982).

The methylation sites in MCP I have been analyzed by trypsin-peptide digest (Kekry and Dahlquist 1982). At least two different tryptic peptides contain methyl esters: One methyl-accepting peptide contains 4 methylation sites and a second tryptic peptide two methylation sites. The tryptic peptide from MCP II has also been shown to have three methylation sites (Chelsky and Dahlquist 1981).

2) MCP methyltransferase and methylesterase: Springer and Koshland (1977), using *S. typhimurium,* observed that methylation of MCP did not occur in a mutant of the generally non-chemotactic cheR class, suggesting the methyltransferase as the *cheR* gene product. This MCP methyltransferase was partly purified from wild type *S. typhimurium* and was shown to have a M, of 38,000. The enzyme was specific for membrane proteins of both *S. typhimurium* and *E. coli.* In *E. coli,* the methyltransferase is encoded by the *cheX* gene.

Recently, MCP methyltransferase has been highly purified from *B. subtilis.* Apparently, in this gram-positive bacteria, there are two types of MCP methyltransferases: a) methyltransferase I (Ullah and Ordal 1981) which methylates membrane proteins from the wild type more efficiently than membranes isolated from a mutant strain defective in chemotaxis.

Table 5. Properties of various protein methylase III's

	Source of protein				
Properties	Calf thymus[5]*	Calf brain[102]	Chicken embryo[103]	Physalum polycephalum[104]	Neuropsora crassa[12]
Subcellular location	Chromatin	Chromatin	Nuclei	Cytosol	Cytosol
In vitro protein substrate	Histone H4	Histones	Histones	Histones	Cytochrome *c*
Residue modified	Res-20				Res-72
Purification achieved (-fold)	100	10	5	40	3,500
Molecular weight		>200,000	~150,000		120,000
pH optimum	7.5-9.0	8.2-8.7	8.4	8.0	9.0
K_m for S-adenosyl-L-methionine (M)	3.0×10^{-6}	1.2×10^{-5}	3.06×10^{-6}	7.3×10^{-6}	1.9×10^{-5}
K_m for protein substrate (M)					7.1×10^{-3}
K_i for S-adenosyl-L-homocysteine (M)		5.7×10^{-6}		70% inhibition at 0.12×10^{-6}	2.0×10^{-6}
Specific activity of the purified enzyme (a)		68	0.38	257	29,500
Requirement for maximum enzyme activity	Mg**		Mg**, dithio threitol	SH reagents	None
Stability	Extremely unstable	6 months at −40°, 90% destroyed in 7 days at 4°C.	50% destroyed at 0°C in 1 month	Destroyed at 60°C for 3 min.	Stable for several months in 50% $(NH_4)_2SO_4$
Ratio of ε-mono:di: trimethyllysine in products		1.95:1.00:0.08		4:1:1	1:3:4

(a) Expressed as picomoles of S-adenoysl-L-[*methyl*-^{14}C] methionine used/min/mg of enzyme protein.
* Reference number.

methylase III				
Saccharomyces crevisiae[105]	Wheat germ[106]	Crithidia oncopelti[107]	Rat brain[108]	Escherichia coli[109]
Cytosol		Cytosol	Cytosol	Ribosome
Cytochrome *c*	Cytochrome *c*	Cytochrome c-557 (Crithidia)	Calmodulin	L11 ribosomal protein of 50S subunit
Res-72	Res-72	Res-2		
63	135			380
97,000				31,000
9.0	9.0	9.0	8.1	7.8–8.2
4.0×10^{-5}	4.8×10^{-5}			
1.33×10^{-3}	6.7×10^{-3}		1.1×10^{-5}	
2.7×10^{-6}				
117	508			2
None	SH reagents		Mn^{++}	Mg^{++}, dithiothreitol
Relatively stable in 50% $(NH_4)_2SO_4$	Quite stable in mercaptoethanol	Stable for 10 days		
9:17:74		Mostly tri-	Mostly tri-, some di- & mono-	Mainly tri-, small amounts of mono-

This enzyme is present in both wild and *che* mutants. Consequently, the role of this enzyme in chemotaxis is uncertain. b) Methyltransferase II (Burgess-Cassler *et al.* 1982), which can be activated by divalent cations, is absent in the chemotactic mutant and, therefore, it is believed to be the enzyme which plays in important role in the chemotactic action.

As a counterpart to the methyltransferase, Stock *et al.* (1978) reported a protein methylesterase from soluble extracts of *S. typhimurium*. This enzyme catalyzes the hydrolysis of γ-glutamyl methyl ester in the MCP, and has a molecular weight of approximately 100,000. It exists as a complex which dissociates into active subunits upon dilution. Analysis of the enzyme activity in a variety of chemotactically defective strains suggests that the enzyme is a product of the *cheX* gene in *S. typhimurium* and the *cheB* gene in *E. coli*. In addition, the authors found that the *cheT* gene product in *S. typhimurium* seems to play a role in the expression of protein methyltransferase activity.

Mutant strains lacking the protein methyltransferase tumble incessantly in the absence of attractant gradients (Stock and Koshland 1978). This behaviour is the converse of that shown by mutant strains defective in protein methylesterase activity, which swim smoothly in the absence of an repellant gradient. This finding, therefore, indicates that the reversible process of protein-carboxyl methylation acts as a control mechanism and that both protein-carboxyl methyltransferase and protein methylesterase are instrumental in the bacterial chemotaxis mechanism.

Enzymology of Protein-Lysine Methylation [Protein Methylase III; EC 2.1.1.43]

Our consideration of S-adenosyl-l-methionine:protein-lysine N-methyltransferase (Protein methylase III; EC 2.1.1.43; the enzyme which catalyzes the transfer of methyl group from S-adenosyl-L-methionine to the ε-amino group of lysyl residues in protein) is complicated by the fact that a wide class of enzymes of this description exist. Their most remarkable characteristics are undoubtedly a high specificity both towards a particular protein as a substrate and moreover particular lysyl residues within that protein (Table 1). The enzyme capable of methylating histone or cytochrome *c* have been most intensively studied, and purified to varying extents from a variety of sources in each case (Table 5). To date, the cytochrome *c* methylases have provided the clearest model of protein-lysyl methylation enzymology and will be considered in this section with a particular emphasis on the enzyme characterized from *Neurospora crassa*.

A. Protein Substrate Specificity.

Among a variety of proteins tested, only cytochromes *c* of various species serve as substrates for *N. crassa* enzyme (Table 6). The variety of cytochromes *c* recognized as substrate are all unmethylated *in vivo* and vary only moderately in substrate capability (40% to 166% relative to horse heart cytochrome *c*). Included in this class are cytochromes *c* from mammals, birds, fishes, amphibians and insects. Yeast cytochrome *c* which *in vivo* is trimethylated at the Res-72 is a non-substrate.

Table 5 lists evidence for the diversity of protein methylase III. Besides the difference between the various preparations with regard to pH optima and molecular weight, etc., the most conspicuous difference is the substrate protein specificity. In addition to the specificity for the species of protein, however, the specificity also extends to discerning among individuals of a similar class protein. For example, two distinct enzymes have been resolved, each specific towards arginine-rich histone H3 or H4 (Sarnow *et al.* 1981; Duerre and Onisk 1982). In the case of *N. crassa*, 3,500-fold enriched protein methylase III methylates only Res-72 lysine of horse heart cytochrome *c* while the 86 position is left unmethylated, thereby mimicking the *in vivo* methylation in *N. crassa*. Since Res-72 and Res-86 lysine residues of wheat germ cytochrome *c* are *in vivo* methylated (Tidwell *et al.* 1968), the above result suggests that there should exist an additional enzyme in wheat germ that recognizes the Res-86 lysine. Recently, a protein methylase III has been partially purified from *Crithidia oncopelti* (Valentine and Pettigrew 1982), and this enzyme is highly specific for its own cytochrome *c*-557 but not for horse heart cytochrome *c*.

Since ε-N-methylated lysines exist in numerous diverse proteins such as flagella protein, myosin, actin, and opsin (Table 2) in addition to histones and cytochrome *c*, it is not unrealistic to expect to find many more pretein methylase III's that are specific for any particular protein.

Table 6. Comparison of the specificity of protein methylase III from calf thymus nuclei with protein methylase III from *N. crassa*

Protein used as a methyl acceptor and source	Enzyme activity (%)	
	Calf thymus	N. crassa
Histone type II-A (mixture)	100[a]	2.9[b]
Lysine-rich histone	94	6.5
Slightly lysine-rich histone	47	0
Arginine-rich histone	162	0
Polylysine	44	0.2
Ribonuclease (bovine pancreas)	6.7	4.7
Albumin	0	0
γ-Globulin	0	0
Cytochrome *c*:		
Horse heart	2.6	100
Rabbit	0	117
Beef	0	112
Guanaco (Llama)		86
Mouse		57
Pigeon		120
Chicken		66
Tuna		96
Frog		40
Silkworm		166
Baker's yeast		4

a. Relative activity based on the substrate histone type II-A as 100% and represents 2.48 p-moles of [*methyl*-^{14}C] transferred/min/mg of enzyme protein.

b. Relative activity based on the substrate cytochrome *c* as 100% and represents 15.3 p-moles of [*methyl*-^{14}C] transferred/min/mg of enzyme protein.

B. Sequence and Structural Determinants for Methylation.

In lieu of the recognition of the Res-72 in native horse heart cytochrome *c* as the methylation site (DiMaria *et al.* 1979), the hemoprotein is treated in various ways and is also cleaved into fragments by cyanogen bromide (CNBr). The K_m and V_{max} values for each of the modified cytochromes *c* and fragments are determined and are compared (DiMaria *et al.* 1979): [thanol-denatured cytochrome *c* (K_m, 0.20 mM) is only a slightly better substrate than the native hemoprotein (K_m, 0.32 mM). Apocytochrome *c* is a much better substrate than either of these (K_m, 0.03 mM) and the residues 1-80 generated by CNBr-treatment is the best substrate overall (K_m, 7 μM). These data have led us to propose that *in vivo* methylation occurs when the protein is nascent or before heme attachment which occurs in the intramembrane space of the mitochondria (Paik *et al.* 1981). The residues 66-104 peptide is a good substrate (K_m, 0.04 mM) while the residues 66-80 is a non-substrate even though it contains the Res-72 methylation site. This result alone with the aforementioned substrate capability of the residues 1-80 suggests that sequence on either side of the 66-80 region is necessary for substrate recognition. It is not known whether this requirement reflect a minimal peptide length or the presence of specific recognition se-

quence. Although a non-substrate, the residues 66-80 peptide is a strong inhibitor of the methylation [at 5μM, it inhibits the methylation of cytochrome *c* (100 μM) by 50%]. The residues 1-65 peptide is a fairly good substrate despite lacking the Res-72 site. The site recognized in this region is lysine 7 and/or 8. This site, however, is not methylated with native, ethanol-denatured, or apocytochrome *c* are used as substrate.

C. Residue Site Specificity

The prufied anzyme from wheat germ of *N. crassa* is shown to methylate native horse heart cytochrome *c* exclusively at the Res-72 which resides in the Asn-Pro-Lys-Lys-Tyr-sequence (DeLange *et al.* 1969). As mentioned earlier, the residues 1-65 peptide from CNBr cleavage of horse heart cytochrome *c* also serves as a substrate: The site of methylation in the polypeptide has been located to a Lys-Lys sequence. Because both of the sites are Lys-Lys-sequence, it is suggested that this sequence is a necessary although not a sufficient factor in site recognition [other Lys-Lys sequence exists in the molecule which is not *in vitro* methylated (DeLange *et al.* 1969)]. However, there are also some exceptions as seen in examples such as in *Crithidia* cytochrome L57,L11 ribosomal protein, and calmodulin (Paik and Kim 1980). These exceptions again emphasize the possibility of individual specific protein methylase III for each protein species.

D. Mechanism of Methylation

In the transfer of methyl groups to cytochrome *c* the enzyme produces a mixture of ε-N-mono-, di and trimethylated lysyl residues in the protein as shown by amino acid analysis of the hydrolyzed methylated cytochrome *c*. The ratio of these methylated lysines produced by the enzyme at each of the various stages of 3,500-fold purification shows very little variation (Durban *et al.* 1978). This lack of ratio variation indicates that a single enzyme or enzyme complex is responsible for the production of these modified amino acid residues.

Time course of production of methylated lysines of horse heart cytochrome *c* as catalyzed by the purified *N. crassa* protein methylase III is shown in Fig. 2. The proportion of trimethyllysine increases with time in relation to mono- and dimethyllysine. This effect becomes especially apparent after incubation for 30-60 minutes. Mono- and dimethyllysine have reached a plateau whereas trimethyllysine is still increasing, reaching a plateau only after 1 hour of incubation. This relative late appearance of trimethyllysine is consistent with a precursor-product relationship whereby monomethyllysine is converted stepwise to di- and trimethyllysine.

The exact mechanism of stepwise methylation is still not clear. However, at least two possibilities can be conceived of: (1) The substrate protein remains

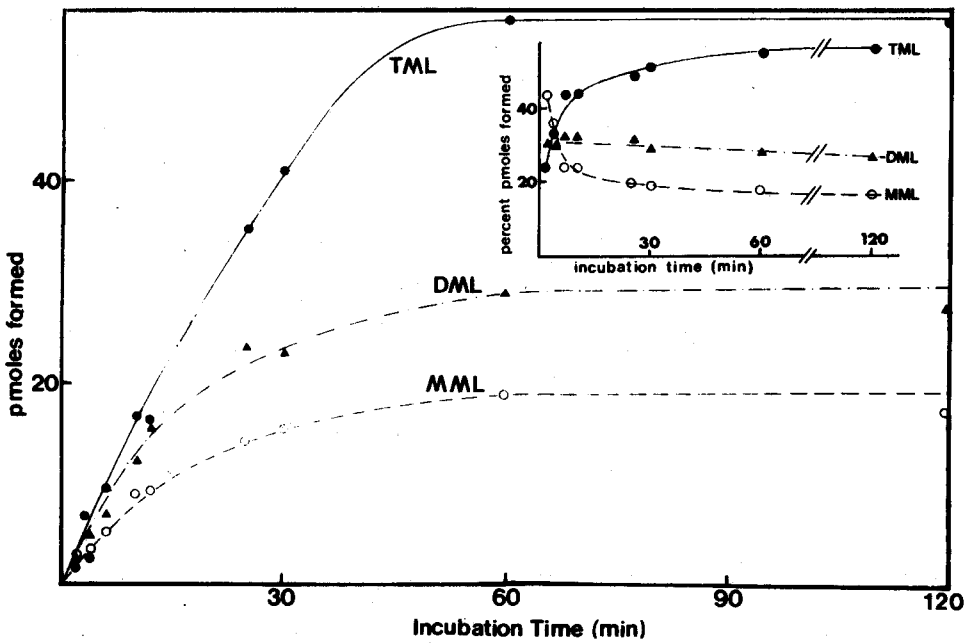


Fig. 2. Time course of formation of methylated lysines catalyzed by cytochrome c-specific protein methylase III (Durban et al. 1978). Amounts of 3-N-mono- (MML), 3-N-di- (DML), and 3-N-trimethyllysine (TML) formed at a given incubatin time were calculated from radioactivity incorporated into the given peaks on amino acid analysis.

bound to the enzyme until it is completely methylated, or (2) once monomethyllysine has been formed, the substrate protein species for further methylation. In the case of *N. crassa* enzyme, possibility (2) is very unlikely, since a vast majority of cytochrome *c in vitro* remains unmethylated·[*in vitro* unmethylated horse heart cytochrome *c* is used as *in vitro* substrate, and more than 99.8% of the substrate remains unmethylated at the end of two hour incubation (Durban *et al.* 1978)]. Therefore, one can envision that the release of monomethylated cytochrome *c* into the large pool of unmethylated cytochrome *c* would make it practically impossible for the monomethylated lysine to become di- and/or trimethylated. The actual experimental finding was quite contrary in that trimethyllysine was formed even at very short incubation time (Fig. 2).

Our preliminary studies indicate that the cytochrome c-specific protein methylase III of *N. crassa* has a ping-pong mechanism (Durban *et al.* 1983). A set of parallel lines was obtained in the initial velocity pattern with cytochrome *c* as the varied substrate. Inhibition by S-adenosyl-L-homocysteine (reaction product) was competitive versus S-adenosyl-L-methionine (methyl donor) and non-competitive versus cytochrome *c*. The results are interpreted to suggest that one group of reactants binds to one site on the enzyme (S-adenosyl-L-methionine and S-adenosyl-homocysteine) and that a second group of reactants binds to a different site (unmethylated and methylated cytochrome *c*), as visualized by a hybride ping-pong mechanism.

E. Biological Significance

1) Histone methylation: Histone-specific protein methylase III activity was found to closely parallel the rate of cell proliferation. Elevated activity was observed in fetal rat brain (Paik *et al.* 1972; Lee and Duerre 1974) fast-growing Morris and Novikoff hepatomas (Paik *et al. 1971b), young rat kidney and testis (Paik et al.* 1971a; Paik *et al.* 1973), continuously dividing HeLa S-3 cell culture (Lee *et al.* 1973), and during hepatic regeneration of adult rat (Lee and Paik 1972). Interestingly there was no change in the enzyme activity in tadpole liver during thyroxine-induced metamorphosis where all biochemical differentiation proceeded in a fixed cell population (Paik and Kim 1975).

Tidwell *et al.* (1968) observed that the rate of histone methylation reaches a peak at 30 hours after partial hepatectomy of adult rat, whereas the rates of RNA and DNA synthesis reach a peak at around 24 hours. A similar result was also obtained with continuously dividing HeLa S-3 cells (Borun *et al.* 1972; Lee *et al.* 1973); very little methylation of histone H3 and H4 occurs throughout the cell cycle until the late S-phase through G_2-phase, where methylation suddenly increased two-fold. Furthermore, if DNA and histone synthesis are inhibited with cytosine arabinoside, the increase in protein methylase III activity still occurs (Lee *et al.* 1973). These result indicate that (1) histone methylation is a late cellular event, and (2) methyllation of histone is not directly related to DNA synthesis (genetic replication) or to RNA synthesis (genetic transcription). This might suggest that histone methylation is involved in chromatic condensation prior to, or during, the process of mitosis (PaiK and Kim 1975).

2) Cytochrome *c methylation:* *Polastro et al.* (1978) earlier investigated the interaction of two forms of yeast *iso*-1 cytochrome *c* (methylated and unmethylated) and cytochrome *c*-depleted mitochondria. Employing three independent methods (oxygen consumption rate, direct binding, and extrinsic fluorescence quenching), they found that methylated cytochrome *c* interacts more strongly with the mitochondria. Thus, enzymatic methylation of Res-72 lysine in *Ascomycetes* cytochrome *c* facilitates its binding to mitochondria. Since cytochrome *c* is synthesized outside mitochondria, its increased affinity toward mitochondria lowers the intracellular level required to give a certain quantity of bound and respiratorily active hemoprotein. This aspect has extremely important consquences for yeast physiology, particularly during a sudden transition between anaerobiosis and aerobiosis. In addition, the selectively higher affinity binding of the methylated species is seen with the homologous yeast mitochondria and not with horse heart mitochondria which could not distinguish between the two forms of yeast cytochromes *c*.

In addition to the above, methylation gives cytochrome *c* protection against intracellular proteolytic enzyme attack. Farooqui *et al.* (1981) studied the *in vivo* stability of methylated and unmethylated cytochrome *c* in *Saccharomyces cerevisiae* after pulse-labeling yeast with [2-^{14}C] methionine and resolving them by isoelectric focusing technique. The rate of unmethylated cytochrome *c* degradation was seen to be three times than methylated cytochrome *c* during the chase period. Furthermore, this protective effect was much less evident in *petite* mutants, which lack high affinity cytochrome *c* binding sites, i.e., functional cytochrome *c* reductase and oxidase, and whose mitochondria are otherwise abnormal. The conclusion derived from the above results is that the mechanism of methylated cytochrome *c* stabilization is best explained by virtue of its higher efficacy of binding import into the mitochondria.

3) Methylation of ribosomal proteins. *E. coli* ribosomal protein L11 contains nine methyl groups out of a total 28 methyl groups so far detected in the RNA and proteins of 50S subunit, and methylation of L11 protein might play an important role in the assembly of 50S subunit. The 50S subunit particles isolated from ethionine grown *E. coli* are defective in association with normal 30S subunit to form 70S ribosomes, are incapable of peptide bond formation, the sedimentation coefficient of these particles is more greatly influenced by Mg^{2+} concentration than the normal 50S subunit, and 23S RNA in the particle is more sensitive to enzymatic degradation (Alix *et al.* 1979). Furthermore, these ethionine grown 50S subunits were found to lack L16 protein and contained reduced amounts of L6, L27, L28 and L30 species. Upon incubation of these ethionine treated bacteria under conditions where methylation of undermethylated cellular components is permitted by the presence of L-methionine but *de novo* protein and RNA synthesis are prevented by rifampicin, however, 50S subunit particles isolated from the above bacteria are fully active in the association with 30S subunit and peptide bond formation. Furthermore, total reconstitution of 50S ribosomal subunits *in vitro* using normal 23S and 55 RNA and proteins prepared from 50S subunits of ethionine treated bacteria yields active subunits only if L16 is also added. Thus, L11 protein methylation appears to be necessary for integration of L16 in the normal 50S assembly process (Alix *et al.* 1979).

4) Protein methylation and carnitine biosynthesis: Carnitine, which is an acyl carrier within the mitochondrial membrane, is synthesized from ε-N-trimethyl-L-lysine in rat liver and *Neurospora crassa*. The formation of ε-N-trimethyl-L-lysine in rat liver results from enzymatic methylation of protein-bound lysine residues by the action of protein methylase III using S-adenosyl-L-methionine and subsequent degradation by hepatic lysosomes. When Chemically ε-N-tri [*methyl*-^{14}C]-labeled asialo-fetuin was administered to rats, it quickly found its way to hepatocyte lysosomes where active proteolytic enzymes rapidly catalyzed the production of free ε-N-tri-[*methyl*-^{14}C] lysine (LaBadie *et al.* 1976). Carnitine was the major radioactive product detected in the extracts of rat carcasses and liver within 3 hours after the administration. Approximately 35% of the ε-N-trimethyllysine present in the administered protein was converted to carnitine. Interestingly, carnitine formation from [*methyl*-^{14}C]-labeled fetuin that contained only ε-N-mono- and ε-N-dimethyllysine residues was not detected even at a point 22 hours after its administration. This finding indicates that only ε-N-trimethyllysine residues in protein are of value for carnitine biosynthesis, whereas ε-N-mono- and ε-N-dimethyllysine residues once freed by proteolysis do not appear to be further methylated to the trimethyl level and thus cannot be utilized for carnitine biosynthesis. The fate of the released ε-N-mono- and ε-N-dimethyllysine may be salvaged as free L-lysine by the emzyme ε-alkyllysinase (EC 1.5.3.4; see next section), which oxidatively demethylates these amino acids, but not ε-N-trimethyl-L-lysine (Kim *et al.* 1964), to formaldehyde and return this essential amino acid to its unmethylated form.

Enzymatic Demethylation of Protein-Bound Methyllysines [ε-Alkyllysinase; EC 1.5.3.4]

The *in vivo* turnover of ε-N-methyl groups in protein-bound methyllysine residues has been described in several systems, such as in histones of HeLa S-3 cells (Borun *et al.* 1972) and perfused cat kidney, (Hempel *et al.* 1979), and in the *iso*-1 cytochrome *c* of yeast (Farooqui 1980).

Only one demethylating enzyme which is capable of removing protein-bound lysyl methyl groups has been described (Paik and Kim 1974). The enzyme is present in mammalian tissues (Table 3), and is especially abundant in the kidney mitochondria. With a high degree of certainty, this enzyme has been shown to be identical to a free ε-N-methyl-lysine demethylase which was originally described by us [ε-Alkyllysinase or ε-Alkyl-L-lysine: oxidoreductase; EC 1.5.3.4) (Kim *et al.* 1964). The enzyme utilizes molecular oxygen and FAD to oxidatively demethylate the methyllysine to form formaldehyde and free lysine residues.

A. Properties

1) Identity of ε-N-methyllysine and histone demethylating enzymes: At each stage of purification of the enzyme from rat kidney the ε-[*methyl*-^{14}C]-l-lysine and [*methyl*-^{14}C] histone demethylating activities are monitored, and the ratio of these two activities remains relatively constant during the course of the purification (130). In addition, the two activities decrease at identical rates upon heating of the partially purified enzyme preparation at 55°C. These results indicate that the same enzyme is responsible for both activities. The enzyme also demethylates free ε-N-dimethyllysine and α-keto-ε-methylaminocaproic acid. However, the one-carbone less analogue, δ-N-methyl-L-ornithine, and ε-N-trimethyl-L-lysine are not demethylated.

2) Various other properties: The enzyme shows optimal activity for demethylating ε-N-[*methyl*-^{14}C]-

L-lysine at pH 7.2 in phosphate buffer, and exhibits classical Michaelis-Menten kinetics with a K_m value of 1.05 mM for ε-N-monomethyl-L-lysine. The divalent cations Ni^{2+} Zn^{2+}, and Co^{2+} are found to be inhibitory at 1.2 mM concentration. At 1.3 mM, KCl reduces the enzyme activity to 40% and 2,6-dichlorophenol is also found to be inhibitory toward the partially purified enzyme.

B. Biological Significance.

As mentioned earlier, turnover of the incorporated methyl groups independent of peptide backbone has been observed in histone of continuously dividing HeLa cells and perfused cat kidney, and in *iso*-1 cytochrome *c* of *S. cerevisiae*. What role, if any, ε-alkyllysinase plays in the turnover of the methyl groups in the above systems is not clear at present. However, ε-alkyllysinase and protein methylase III, which methylates the ε-amino group of lysine residues, show an opposing pattern of activity in various physiological conditions (Paik and Kim 1974). The ε-alkyllysinase activity in the liver and Kidney of a young rat is relatively low are reaches the adult maximum level during or before puberty, whereas protein methylase III activity is high in young rat liver and kidney and decreases to the adult minimum level in a pattern opposing that of ε-alkyllysinase (Fig. 3). This opposing pattern of alteration in protein methylase III and ε-alkyllysinase activity is also found in the fast-growing Novikoff hepatoma; where ε-alkyllysinase activity is practically negligible in the hepatoma, protein methylase III activity is very high. Since these two enzymes most probably possess biochemically opposite functions, the pattern of change in the enzyme activity is a strong indication of a possible turnover mechanism of the incorporated methyl groups.

CLOSING REMARKS

Protein methylation is a complex biochemical reaction occurring ubiquitously in prokaryotic and eukaryotic organisms. Since the first discovery of the presence of methylated lysine in the flagella protein of *Salmonella typhimurium* 24 years ago, there has been tremendous progress in the area of chemistry and enzymology of protein methylation. Since 1976, the biologic significance of proetin methylation has begun to emerge, and the finding of a precursor-product relationship for protein methylation and carnitine biosynthesis is the first solid demonstration of biologic importance of protein methylation. The observation that cytochrome *c* methylation facilitates the binding of this hemoprotein to mitochondria could be placed as the second example along this line. Furthermore, the protein-carboxyl methylesterification has been suggested to be a crucial factor in chemotaxis in bacterial systems, and evidence for this suggestion is compelling. In adition to these examples, the methylation of ribosomal proteins, opsin, flagella protein, calmodulin, HnRNP protein, histones, heat-shock protein, elongation factor, α-amylase, citrate synthase, and myelin basic protein offer potentially fertile areas of research endeavor.

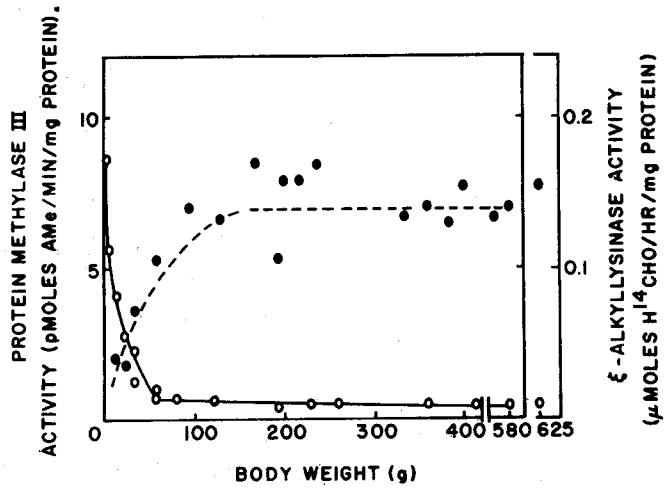


Fig. 3. Changes of protein methylase III and ε-alkyllysinase activity in the rat kidney during growth. Open circles represent the activity of protein methylase III and closed circles that of εalkyllysinase.

The recent discovery of highly cytochrome *c*-specific protein methylase III from fungi and plant has opened a new horizon in the enzymology of protein methylation. This indicates that the recognition of ε-amino group of lysyl residues as the site of methylation is not the only requirement for specificity exhibited by the enzyme. An additional level of specificity is also required in the identity of the methylacceptor protein. Thus far, three distinctly different protein species-specific protein methylase III's are identified; histone-, cytochrome *c*-, and calmodulin-specific. Similarly, histone- and MBP-specific protein methylase I's have also been identified. Therefore, it is highly realistic to expect to find many more protein methylases that are specific for particular proteins.

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